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(71) Applicant *(for all designated States except US)*: THE PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; University Place, 124 Mount Auburn Street, Cambridge, MA 02138 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ROWITCH, David, H. [US/US]; 171 Holworthy Street, Cambridge, MA 02138 (US). McMAHON, Andrew, P. [GB/US]; 128 Kendall Road, Lexington, MA 02421 (US).

(74) Agent: STRIMPEL, Harriet, M.; Bromberg & Sunstein LLP, 125 Summer Street, Boston, MA 02110–1618 (US).

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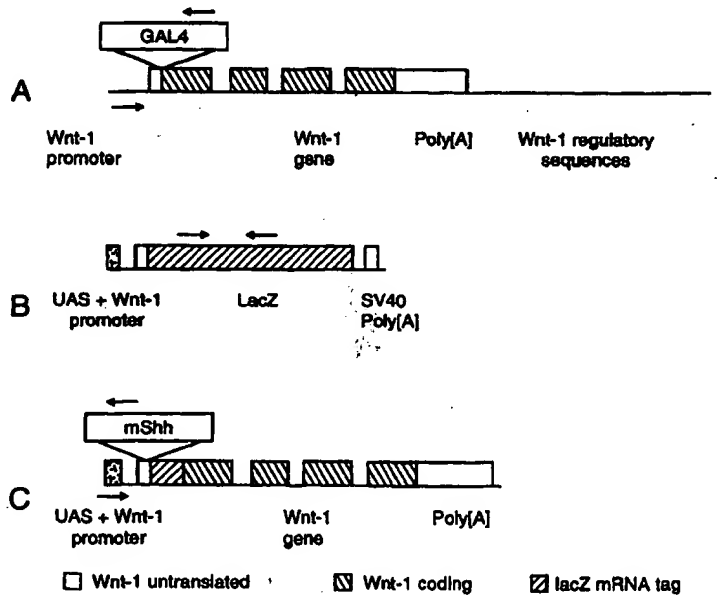

(57) Abstract

Transgenic stable animal lines having genes essential for development under regulation of extra–mammalian transcription signals, such that bigenic combinations cause ectopic expression of the transgene in one or more specific tissues, and methods of use of bigenic animals and cells, are provided.

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USE OF BIGENIC MOUSE MODELS AND ASSAY SYSTEMS TO IDENTIFY AGENTS THAT REGULATE PROLIFERATION AND DIFFERENTIATION

Technical Field

The present invention relates to bigenic mouse lines, cells of the mouse lines, and methods of use to identify agents that modulate the activity of cell proteins involved in tissue proliferation and differentiation.

Background

In developmental biology, a dominant lethal mutation that occurs in germ cells or is introduced into the fertilized egg using transgenic methodology can result in death of the embryo early in gestation. The death of the embryo is problematic with regard to analysis of the defect because it is not possible to form breeding colonies of these mutants. A similar problem can arise when a transgenic mouse is made in which a protein is expressed in abnormal amounts or in unusual tissues so as to give rise to a lethal phenotype. Consequently, a new transgenic manipulation is required each time such effect in the protein is to be studied which adds to the cost, the time and the variability of the experimental system.

Improved animal model systems would be desirable for studying the developmental consequences of a lethal phenotypes in the embryo in utero and for providing a screening system for identifying novel therapeutic agents that may be used to correct the resultant abnormalities in the adult animal.

Among proteins that play an important role in development are the class of regulatory molecules identified as hedgehog proteins. The hedgehog family of proteins, which was first identified in *Drosophila* and subsequently observed in mammals, is expressed in different organizing centers which can initiate signals that pattern neighboring tissues. In particular, hedgehog proteins play an important role in the development of the central nervous system including forebrain development.

There are at least eight cloned hedgehog proteins having a broad spectrum of biological activity (WO 95/18856). The biological activities of the hedgehog proteins have been described in Hammerschmidt et al. *Trends in Genetics* (1997) 13:14-21 and in Tabin C, et al. *Trends in Cell Biology* (1997) 7:442-446, both references hereby incorporated by reference herein. Defects in hedgehog genes and hedgehog receptor genes have been correlated with several human diseases (Hammerschmidt et al., 1997). These include cancers such as brain tumors including medulloblastoma and meningioma; skin cancers such as basal cell carcinoma; and breast cancer.

There is a current lack of animal models for analyzing the effects of defective regulatory genes during development and for providing screening assays to identify therapeutic agents that might regulate the function of these regulatory proteins.

Summary of the Invention

In a preferred embodiment of the invention, a transgenic non-human mammal is provided, substantially all of whose cells contain a non-viral regulatory DNA sequence linked to a recombinant hedgehog gene introduced into the mammal or an ancestor of the mammal at an embryonic stage. For example, a transgenic non-human mammal can be the mammal having an endogenous coding sequence substantially the same as a coding sequence of the recombinant hedgehog gene. In one embodiment of the invention, the mammal is a rodent, for example, a mouse. In a preferred embodiment of the transgenic non-human mammal, the regulatory sequence comprises a UAS sequence (SEQ ID NO:1).

In a further embodiment the invention provides a bigenic non-human mammal, substantially all of whose cells contain a non-viral regulatory DNA sequence linked to a recombinant hedgehog gene sequence; and a transcriptional activator sequence, introduced into the mammal or an ancestor of the mammal at an embryonic stage. Thus the embodiment can be a bigenic mammal having an overexpressed vascular system in the central nervous system, for example, the mammal is an embryo, and is capable of a lifespan, for example, of 9 dpc, of 12.5 dpc, of 18.5 dpc, of parturition, of one month post-parturition, of three months post-parturition, or of one year post parturition.

A further embodiment of the invention is the bigenic non-human mammal wherein the transcriptional activator gene is *GAL4*. Further, the transcriptional activator gene is regulated by a tissue specific promoter, for example, the tissue specific promoter is a *wnt*

promoter or a *col II* promoter. In a further embodiment of the bigenic non-human mammal, the transcriptional activator gene is regulated by an inducible promoter, for example, the inducible promoter is regulated by a fusion of *GAL4* protein and a second protein, for example, the second protein is activated by binding an RU486 mifepristone molecule.

A further embodiment of the invention is a bigenic non-human mammal for use as a model for disease, for example, the disease is cancer, for example, a cancer of the breast, skin, prostate, kidney, lung, and central nervous system. In a preferred embodiment, the cancer is a primitive neuroectodermal tumor, or the cancer is a medulloblastoma.

A further embodiment of the invention provides an isolated cell of a bigenic non-human mammal obtained from the above bigenic mammal. The isolated cell of the bigenic non-human mammal is selected from the group consisting of an embryonic-stem cell, a tumor cell, a nerve cell, and a vascular cell.

Another embodiment of the invention is a transgenic non-human mammal having an insertion mutation of an *Ihh* gene, for example, the insertion can comprise a selectible marker, and the insertion can comprise a deletion of the *Ihh* gene.

A further embodiment of the invention provides an isolated population of cells selected from the group consisting of a transgenic non-human mammal, and its bigenic progeny.

In another aspect, an embodiment of the invention is a method of identifying the effect of misexpression of a target transgene in a signal transduction pathway that includes a hedgehog protein, in a progeny animal, comprising: (a) forming a first transgenic animal having a first transgene encoding a transcriptional activator of a eukaryotic species different from the animal; (b) forming a second transgenic animal having a second transgene comprising the target gene and having a recognition sequence for the transcriptional activator that is located upstream of the target gene; (c) mating the first and the second transgenic animals to form a bigenic animal; and (d) causing the target gene to be misexpressed in the animal. A further aspect of this method of identifying the effect of misexpression of a target transgene provides the transgenic animals formed from an animal which is an animal model disease line, for example, the animal model is selected from the group consisting of a cancer and an autoimmune disease.

A further embodiment of the invention is a method of assaying for a temporal

requirement for the presence of a hedgehog protein on progression of a disease, comprising:

(a) forming a bigenic animal according to the method above; (b) treating the bigenic animal for an effective time interval with an agent that interrupts the hedgehog pathway; and

(c) assaying the progression of the disease in the animal in (b) compared to the progression of the disease in the animal in (a).

Another embodiment of the invention is method of assaying for a temporal requirement for the presence of a hedgehog protein during progression of the disease in the bigenic animal in the disease model line, wherein the treatment comprises administration of an agent selected from the group consisting of an inhibitor of cholesteroid biosynthesis, an anti-hedgehog antibody, and a sterol analog.

An embodiment of the invention further provides a method for determining therapeutic efficacy of an agent, comprising: (a) forming a bigenic mouse according to the method above, wherein the misexpressed target gene is a hedgehog gene; (b) administering the agent to the mouse; and (c) determining therapeutic efficacy of the agent. Another method provides that (b) further comprises administering the agent to the mouse in a pharmaceutical carrier at an effective dose. Yet another method provides that (c) further comprises comparing lifespans of the bigenic mouse of (b) with the bigenic mouse of (a), for example where the bigenic mouse in (a) is an embryo.

A further embodiment of the invention is a method of obtaining an expanded population of neural stem cells from a subject, comprising: treating a neural stem cell from the subject with a hedgehog protein, so that proliferation of the stem cell provides an expanded population of neural stem cells. In a preferred embodiment of the method, the hedgehog protein is sonic hedgehog protein. In another embodiment of the method, the subject has a condition selected from the group consisting of Parkinson's disease, Alzheimer's disease, and spinal cord injury. Thus the condition can be treated by administration to the subject of a sample of the expanded cell population.

An embodiment of the invention provides a method for inactivating an *Ihh* gene in a non-human mammal, comprising: (a) constructing a recombinant vector carrying an *Ihh* insertion mutation: (b) injecting an embryonic stem cell with the vector; and (c) implanting the stem cell into an adult mammal. In a preferred embodiment of this method

for inactivating an *Ihh* gene, the vector in (a) carries a deletion of exon 1 of the *Ihh* gene.

Brief Description of the Figures

Figure 1 is a schematic representation of a signaling pathway of which exogenously supplied hedgehog functions to effect changes in nuclear gene transcription. HH, hedgehog protein is shown as an extracellular protein that binds a receptor composed of the products of genes indicated PTC (for *patched*), and SMO (*smoothened*). In the absence of HH binding, a cytoplasmic protein complex comprising microtubule protein and Cos-2 (costal-2) and Fu (Fused), functions with a repressor form of CI (cubitus interruptus), indicated as Rep.-CI= transcriptional repressor. In the presence of HH binding to the receptor, the protein complex dissociates from the microtubule protein, and as a result of a series of protein phosphorylations, an activator form appears, indicated Act.-CI (transcriptional activator). The activator or repressor form of CI is translocated into the nucleus where it regulates transcription of the HH target family of genes, in the activator form complexed with CBP (CREB binding protein).

Figure 2 shows a schematic drawing of transgenes WEXP-*GAL4*, UAS-*lacZ* and UAS-*Shh* used in the bigenic system for misexpression in the mouse embryonic CNS. (A) Plasmid pWEXP-*GAL4* is a driver vector that comprises full-length *GAL4*, which was cloned into the WEXP2 expression vector under control of the *Wnt-1* promoter. (B) The reporter transgenic construct, a vehicle vector carrying a UAS-*lacZ*, was constructed to utilize the *Wnt-1* promoter and 5 copies of the UAS. (C) Plasmid pUAS-*Shh* was constructed with full-length mouse *Shh* cDNA target gene cloned into expression vector WEXP3C. *Wnt-1* regulatory sequences were then replaced by 5 copies of the UAS. The arrows indicate sites of hybridization for each of the oligonucleotide primers used in genotyping the various transgenic lines and progeny as described in the Examples.

Figure 3 is a diagrammatic representation of the strategy for forming mouse *GAL4/Wnt-1* and *lacZ* reporter transgenic mouse lines suitable for crossing to obtain bigenic progeny. Blue color indicating *GAL4* mediated Wnt-1 promoted expression of *lacZ* was visible in the mouse embryo at the dorsal region of the neck.

Figure 4 shows the *GAL4*/UAS bigenic system for misexpression of proteins in transgenic (Tg) mice, wherein (a) shows structure of the WEXP-*GAL4* transgene; (b) shows structure of the UAS-*lacZ* transgene and expression of β-galactosidase in the Wnt-1 pattern in a *GAL4*/UAS-*lacZ* 10.5 dpc (days post coitum) embryo with black arrows

indicating expression observed in the midbrain-hindbrain junction and dorsal spinal cord as a blue color; and (c) shows structure of the UAS-*Shh* (sonic hedgehog) transgene and phenotype of a *GAL4*/UAS-*Shh* 10.5 dpc embryo. Ectopic expression of the neural tube floorplate marker, HNF3β is indicated by arrows and occurs in analogous positions to *lacZ* expression under wnt-1 in the brain (b).

Figure 5 shows data obtained from the *GAL4*/UAS system for providing ectopic gene expression in the developing CNS. Panels A-D show whole mount histochemical analysis of target reporter β-galactosidase activity in transgenic mouse embryos. A: Lateral view showing pattern of *lacZ* expression under control of *Wnt-1* regulatory sequences. B, C: Lateral views of 10.5 dpc and 12.5 dpc bigenic *Wnt-1-Gal4 X UAS-lacZ* embryos showing expression pattern of *lacZ* (arrow indicates roofplate expression in the spinal cord). D: Transverse (top) and bisected (bottom) views of *lacZ* expression in the rostral spinal cord of a bigenic embryo at 18.5 dpc. Note staining in roofplate oligodendrocytes that project to the ventricular zone (arrows). Panels E-J show morphological analysis of wild-type (left) and *Wnt-1-GAL4 X UAS-Shh* bigenic (right) litter mates at: E: 10.5 dpc (arrow indicates anterior neural tube defect); F: 12.5 dpc. Panels G-J show analysis at 18.5 dpc. Lateral views are shown of (G) wild-type and (H) bigenic embryos. Note tissue mass protruding from midbrain which covers cerebral hemispheres (arrow). Panel I: Dorsal view of bigenic embryo showing hyperplastic spinal cord which protrudes from the back covered by a thin epithelial membrane. Note prominent vasculature and hemorrhage (arrow). J: Dorsal view of skeletal preparation of wild-type (left) and bigenic (right) embryo at 18.5 dpc. The membranous skull and dorsal neural arches were observed to be absent, and the vertebral bodies displayed a splayed open configuration (arrows) as a result of ectopic *Shh* expression.

Figure 6 demonstrates hyperplasia in the dorsal central nervous system (CNS) of *Wnt-1-GAL4 X UAS-Shh* (referred to as UAS-*Shh* Tg) bigenic mice, wherein (a) shows a dorsal view of a 18.5 dpc UAS-*Shh* Tg embryo demonstrating overgrowth of the brain and spinal cord, and increased vasculature in the dorsal CNS where *Shh* is ectopically expressed; and (b) shows a transverse section at the level of the forelimb of an 18.5 dpc UAS-*Shh* Tg embryo. The region dorsal tissue in the spinal cord was observed to be hyperplastic, with hydromyelia (gross enlargement and distension) of the spinal cord central canal.

Figure 7 shows the result of misexpression of *Shh* in the dorsal CNS. Ectopic expression of *Shh* in the midbrain ventralizes the midbrain, inducing ectopic expression of *Shh* target gene Hnf-3β in the dorsal CNS. The whole mount was analyzed by Hnf-3β immunostaining.

Figure 8 shows the targeting strategy used to generate *Ihh* mouse knock-out mutants by homologous recombination with a vector in which the first exon at the *Ihh* locus is deleted, thereby generating a null allele.

Detailed Description of Embodiments

We have examined the effect of misexpression of proteins that play a role in the developing embryo. In particular, we have examined the effect of misexpression of hedgehog proteins, more particularly Sonic hedgehog proteins (*Shh*), using bigenic mouse models and the effect of loss of function of Indian hedgehog in transgenic mouse embryos.

Hedgehog

A hedgehog protein includes a member of a family of cell proteins found in invertebrates such as *Drosophila* and in vertebrate eukaryotes, including humans, which are essential to tissue pattern formation that distinguish the variety of tissues formed during embryonic development. A hedgehog protein molecule is post-translationally modified by addition of a cholesterol molecule. Figure 1 shows the hedgehog signal transduction pathway and the concomitant effects on nuclear transcription of binding of a hedgehog molecule to a cell receptor.

Hedgehog proteins are secreted factors and determinants of dorsal-ventral polarity in the central nervous system (CNS), being essential for induction and subsequent differentiation of ventral cell types. Hedgehog proteins act via signal transduction. Sonic hedgehog appears to induce hypervascularization, hyperplasia and in some situations, neoplasia, a property that is shared with Indian hedgehog protein which appears however to reduce vascularization. Neoplasia is linked not only to hedgehog gene expression but also in humans to the loss of the *patched* protein receptor (Figure 1). *Patched* is a membrane protein which down regulates transcription of the genes encoding transforming growth factor (TGF) β and *Wnt* families, and transcription of its own gene. *Patched* has been implicated in oncogenesis, including development of basal cell carcinoma and of nevoid basal cell carcinoma syndrome (Gorlin, (1987) *Medicine* 66:98-113), which

includes fibromas of the ovaries and heart, cysts of skin and jaw and mesentery, meningiomas and medulloblastomas.

Until now, the effects on the embryo, particularly the proliferative effects, of expression of hedgehog proteins in abnormal amounts or in tissues that do not normally express such proteins have remained incompletely understood. The present invention provides a binary transgenic (bigenic) system in which protein expression exemplified by expression of hedgehog is regulated by a promoter that is not normally recognized in a mammalian cell (described herein by the term "extra- mammalian" promoter; Figures 2,3). The promoter is in turn activated by a transcriptional activator protein, expression of which is under the control of a tissue specific enhancer. A binary transgenic system provides the opportunity to activate otherwise silent transgenes in progeny obtained from a simple genetic cross, because the transcriptional activator is maintained in one line of mouse, while the silent target gene under control of an extra-mammalian promoter is maintained in a second mouse line. Only when the two lines are crossed does abnormal expression of the target gene occur (A. Brand et al. (1993) *Development* 118:401-415; Ornitz et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:698-702). Ornitz et al. utilized a bigenic system to study the effects of misregulating *int-2*, using a mouse mammary tumor virus (MMTV) promoter to transcribe the activator protein gene. The *int-2* gene is expressed in mammalian cells, and is implicated in mammalian neoplasia. In the Ornitz system, tumors resulted from ectopic expression of *int-2* only in the mammary and salivary glands. Brand et al. created a bigenic system in *Drosophila* to study the homeobox protein, *even skipped.*

The term hedgehog as used here and in the claims shall include the hedgehog protein from any organism, for example from an invertebrate or a vertebrate organism, and shall include any polypmorphic variant or mutation, including a substitution mutation, a deletion, or an insertion mutation that retains the developmental and differentiation functions of dorsoventral patterning and proliferative function of the wild type hedgehog protein. The term shall further include hedgehog proteins of all members of the hedgehog family, for example, Sonic hedgehog, Indian hedgehog, desert hedgehog, zebra hedgehog, tiggywinkle, and other members of the hedgehog family. The hedgehog protein of the invention shall include a hedgehog protein synthesized from a hedgehog-encoding DNA sequence that is at least 70% homologous, at least 80% homologous, at least 90%

homologous, at least 95% homologous, and at least 98% homologous to the sonic hedgehog protein which retains the functions of the hedgehog protein. The hdegehog protein includes a protein encoded by a nucleic acid that hybridizes under stringent conditions as defined herein with a portion of a gene encoding a hedgehog protein. The term shall further include any chemical analog or derivative composition of a hedgehog protein, including a peptidomimetic which maintains the hedgehog functions as described in the examples herein.

Hedgehog is a secreted protein that functions as modified by addition of a cholesterol moiety as an intercellular signalling system. Hedgehog signalling can be interrupted by one of several chemical treatments of an animal or a cell in culture, for example, by addition of an anti-hedgehog monoclonal or polyclonal antibody, by addition of an inhibitor of cholesterol biosynthesis such as lovastatin, pravastatin and simvastatin (Merck, Rahway, NJ), or by addition of the steroidal alkaloid cyclopamine (Incardona, J. et al.(1998) Devel. 125:3553-3562). Cyclopamine has been shown to exert teratogenic effects (for example, cyclopia) due to direct antagonism of sonic hedgehog signal transduction. Administration of these agents to a bigenic animal that is an embodiment of the invention, particularly bigenic animals that are formed in an animal line that is an animal model of a disease, at times during embryonic development, can provide a method of assaying for a temporal requirement for hedgehog protein during the progression of a disease.

Definitions

As used in this description and in the accompanying claims, the following terms shall have the meanings indicated unless the context otherwise requires.

The term "protein" includes the terms "polypeptide," and "peptide."

A "regulatory DNA sequence" is used herein to describe a sequence of DNA to which one or more proteins can bind, so that transcription of a DNA sequence can be initiated or increased or decreased. The regulatory DNA sequence can be a promoter, an operator, or an enhancer site. A regulatory DNA sequence can also be a terminator site, for example, a tract of several adenyl residues at the end of a gene (polyA).

A UAS is an upstream activating sequence that can bind a *GAL4* protein, such binding resulting in an increase in transcription of DNA downstream from that site. An enhancer site can also increase transcription from downstream DNA. The UAS of the

invention (SEQ ID NO:1) is known to function from a location that is upstream of the target gene.

A "promoter" is a DNA sequence with ability to bind to an RNA polymerase molecule to initiate transcription. Extent of binding is influenced by promoter strength, and by protein factors that interact with an enhancer site at or adjacent to the promoter.

A regulatory DNA sequence can be located within a protein coding region of a gene, however, the protein coding region of all or a part of a gene shall be referred to herein as a gene. The engineered vectors used herein generally select a site upstream of a gene for regulation of transcription of the gene, however it is within the scope of the invention to locate a regulatory sequence within the protein coding region of a gene.

A "recombinant protein" is a protein that is synthesized in vivo from a transgene or a recombinant gene, so that it can be distinct both in cell location and in regulation of expression from a naturally occurring homologous gene, if any, within the cell. The recombinant proteins that are used in embodiments of the present invention can carry mutations, including without limitation, substitutions, chain terminations, deletions and insertions. Further, a recombinant protein can be encoded by DNA that is homologous to the gene encoding the protein, providing that the function of the protein is retained.

A "bigenic" animal is the result of a cross between two different transgenic animals, such that a 1:1:1:1 Mendelian progeny ratio is observed, the ratio describing progeny which consist of one wild type, one of each of the single hemizygous transgenic animals, both of which may have phenotypes identical to the wild type, and one bigenic progeny animal. Figure 2 shows vectors for forming transgenic animals, which can be combined by a genetic cross to obtain bigenic progeny. Figure 2A shows a "driver vector" capable of producing *GAL4*, a transcriptional activator protein native to yeast *Saccharomyces cerevisiae*. This vector confers no phenotype itself in a mammalian cell, as *GAL4* is "extra-mammalian" and therefore does not interact with the wild type mouse genome. Figure 2B and 2C show "vehicle" or "carrier" vectors which can express a "target" gene carried on the vehicle, but only in the presence of *GAL4* protein, at the time it is synthesized by expression of the *GAL4* gene on the driver vector.

"Temporal requirement" indicates a window of time of development during which a specific factor must be present. The temporal requirement may be a period of transient gene expression, for example, from the period between 9.5 and 12.5 dpc, or may be of

long duration, for example, from conception, or from 4.5 dpc, up until parturition or even beyond.

An "embryo" of an animal is the term used to describe the progeny from the zygote stage until parturition.

An "inducible" gene is capable of being expressed in response to addition to cells or administration to an animal of an exogenous chemical or drug. Transcription mediated by a steroid-receptor like protein to which the chemical RU486 (mefipristone; Roussel-Uclaf; Hoechst) has been bound is inducible by administration to an animal or addition to cells in culture of this chemical.

A "fusion" protein is a non-naturally occurring protein obtained from genetic manipulation of two or more genes encoding respectively amino acid sequences derived from two or more different proteins, to create a fusion gene having the two or more proteins tranlated in the same reading frame. In an embodiment of the present invention, the *GAL4* protein can be fused to all or a portion of another protein, for example to confer inducibility, such that mifepristone (RU486) can be added to cell medium or administered to a transgenic or bigenic animal to induce expression of another gene.

"Homology" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous or identical at that position. A degree of homology between sequences is a function of the number of matching or identical positions shared by the sequences.

A "gene" encoding for a hedgehog protein, for example a sonic hedgehog or an Indian hedgehog protein within the scope of an embodiment of the invention if it encodes a protein that has substantially the same function of the wild-type hedgehog protein. A hedgehog gene that is within the scope of the present invention may have a mutation, for example without limitation, a point mutation resulting in an amino acid substitution or chain termination, a deletion, an insertion, if the encoded protein retains hedgehog function. Further, a gene that is homologous to a hedgehog gene that encodes a protein capable of conferring a normal hedgehog phenotype is within the scope of the embodiment of the invention.

Preferred regulatory sequences encode a UAS sequence which is at least 76.4%

homologous (having 13 homologous and 4 nonhomologous nucleotide residues), more preferably at least 82.3% homologous (having 14 homologous and 3 nonhomologous nucleotide residues), more preferably at least 88.3% homologous (having 15 homologous and 2 nonhomologous nucleotide residues), and even more preferably at least 94.1% homologous (having 16 homologous and 1 nonhomologous nucleotide residues). A *GAL4* protein, which is an embodiment of the invention is one comprising an amino acid sequence which retains the functions of binding to a UAS and activating transcription, and has an amino sequence which is at least 60% homologous, more preferably 70% homologous and most preferably 80%, 90%, or 95% homologous with the wild type *GAL4* amino acid sequence (Brand, A. et al., 1993).

Another aspect of the invention provides a nucleic acid which hybridizes under high stringency conditions to a "probe", which is a nucleic acid which encodes a portion of an inserted transgene sequence as shown in SEQ ID Nos: 2, 3, and 4. A suitable probe is at least 12 nucleotides in length, is single-stranded, and is labeled, for example, radiolabeled or fluorescently labeled. Appropriate moderate conditions of stringency of conditions of formation of double-strandedness which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, are followed by successive washes of increased stringency, e.g., 2.0 x SSC at 50°C, and are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Other suitable stringency conditions include selecting the salt concentration in the wash step from a low stringency of about 2.0 x SSC at 50°C, and then using a wash of a high stringency condition, of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. A *GAL4* protein which is an embodiment of the invention is encoded by a gene which hybridizes to the wild type *GAL4* gene under stringent conditions.

Conditions for hybridizations are largely dependent on the melting temperature for half of the molecules of a substantially pure population of a double-stranded nucleic acid, a parameter known as the T_m. For nucleic acids of sequence 11 to 23 bases, the Tm can be calculated in degrees C as 2(number of A+T residues) + 4(number of C+G residues). Hybridization or annealing of the probe to the nucleic acid being probed should be conducted at a temperature lower than the Tm, e.g., 15°C, 20°C, 25°C or 30°C lower

than the Tm. The effect of salt concentration (in M of NaCl) can also be calculated, see for example, Brown, A., "Hybridization" pp. 503-506, in *The Encyclopedia of Molec. Biol.*, J. Kendrew, Ed., Blackwell, Oxford (1994).

An "animal model" for a disease is an animal treated with a chemical composition, or a mutant animal, that displays symptoms identical or similar to a human subject having the disease. The mouse mutant *Patched*, for example, is an animal model of cancer. Animal models for human cancers can be formed using the bigenic animals that are embodiments of the present invention, for example, misexpression of a hedgehog protein causing ectopic proliferation of nerve stem cells in the CNS can be an animal model of a brain tumor.

A "therapeutic effect" resulting from addition to a cell culture or administration to an animal of a chemical or a protein agent can be observed as a prevention or a remediation of symptoms of disease, in comparison to cells or animals not receiving the chemical. For remediation or prevention of symptoms in the present invention, a therapeutic effect is a demonstration of effectiveness of an agent to cause a phenotype which is more normal than a phenotype observed from ectopic expression of a hedgehog protein, for example, in the bigenic animals of the invention.

An "effective dose" is that amoung of exogenously added or administered, or in vivo generated *Shh* protein or other hedgehog protein, or other chemical entity, capable of achieving a successful endpoint of a therapeutic effect.

Advantages of the bigenic system include reduction in cost and time compared with forming transgenic animals de novo by micro-injection and genotypic screening prior to performing each experiment. Large numbers of bigenic embryos can be generated by cross breeding. Bigenic transgenic systems may be formed as follows: a first or "carrier" vector is made having a regulatory sequence placed adjacent to and upstream of a target gene of interest. The regulatory sequence is recognized by an extra-mammalian regulatory protein, for example the extra-mammalian upstream sequence may be a regulatory sequence from yeast (for example, UAS) or from herpes virus (for example, IPE). A second or "driver" vector contains DNA encoding a gene for a transcriptional activator wherein the expression of this gene is capable of triggering the regulatory sequence in front of the target gene on the first carrier vector, for example yeast transcriptional activator (*GAL4*), or herpes transcriptional activator (VP16).

A tissue specific regulatory sequence may be used to promote the expression of the transcriptional activator such as *wnt-1* enhancer and promoter which target gene expression to cells of the nervous system (Figures 2, 4). The *Wnt-1* enhancer is ideally suited for directing gene expression in the roofplate of the CNS (Echelard et al., *Development* (1994) 120:2213-2224), and it has been used to misexpress chicken *Shh* in transgenic mice (Echelard et al., *Cell* (1993) 75:1417-1430); however in studies by Echelard et al. (1993, 1994) the CNS malformation resulting from *Wnt-1* control of *Shh* expression was lethal, and no transgenic embryos survived to birth.

Other tissue specific regulatory sequences may be used, including those that target bone (for example collagen type II enhancer), skin (keratin-14 enhancer), kidney (*pax-2* enhancer), and CNS (nestin, neuron specific enolase, transerythrin). These tissue specific promoters may in turn be regulated by inducible enhancer sequences. An example of an inducible system utilizes RU486 which binds to a membrane receptor protein and is translocated to the nucleus (Wang et al., *Nature Biotechnology* (1997) 15:239-243) and therefore has particular utility for regulating expression of hedgehog protein. Another example of an inducible system having utility in this invention is a tetracycline regulated system in which the tet repressor from *Escherichia coli* is fused to the Herpes simplex virus viral protein 16 (VP16) transcriptional activation domain (Schockett et al. *Nature Biology* (1997) 15:217) such that addition of tetracycline induces VP16-promoter regulated expression.

The present invention uses a regulated gene expression system to cause selective expression of hedgehog proteins, for example Sonic hedgehog protein (*Shh*), and also to express *lacZ* as a convenient marker ("reporter") system, well known in the art, to monitor the activity of enhancers, promoters and transcriptional activator sequences. The *lacZ* reporter is used as a control in parallel with studies on hedgehog gene expression (Figures 2-4).

Each of two types of vectors is introduced into a mouse to yield two types of lines of novel transgenic mice, each having a normal phenotype. The target gene is not expressed until the two mouse strains are cross bred and progeny embryos are obtained. The progeny of the cross can express the target gene, and therefore the phenotype observed in the progeny of the cross is different from that of each of the two parental lines. Because the extra-mammalian transcriptional activator exemplified by *GAL4* is not

normally present in mouse cells, genes with lethal effects on the embryo can be stably maintained in transgenic mice under control of the extra-mammalian promoter (exemplified by UAS). An embodiment of the invention uses this novel system to determine the effects of synthesis of abnormal amounts of regulatory proteins during embryogenesis.

The bigenic mouse model as used herein illustrates the proliferative effects of Sonic hedgehog protein in the CNS of animals as exemplified by the following:

(a) CNS hyperplasia was analyzed to determine the constraints, if any, on competence to respond to Hedgehog signaling by neural cells;

(b) The impact of *Shh* on patterning and cellular induction was determined. Using the bigenic system embodiment of the invention, it was here observed that abnormally high levels of Sonic hedgehog expression resulted in increased vascularization in the nervous tissue of the brain and the dorsal CNS (Figures 4-6).

In the novel transgenic mouse progeny, unexpected changes in vascularization of the nervous system were observed herein to arise when hedgehog proteins were defective. The bigenic mouse provides a system for analyzing *in vivo* vascularization of the brain, and an assay system to identify novel factors to enhance or diminish such vascularization. Furthermore, an embodiment of the invention provides explant cultures of the hypervascularized tissue, which when cultured *in vitro*, provide a system for analysis of translocation of drugs across the blood brain barrier, for identifying agents that affect translocation, for analyzing hyperplastic including neoplastic properties of the cells, and for analyzing agents that modulate or reverse the hyperplasia.

An embodiment of the invention provides a system in which for the first time abnormal activation of hedgehog signal transduction in specific neural cells can be associated with generation of neoplasia. Further, over-expression of *Shh* in the brain unexpectedly caused the surface of the mouse brain, usually smooth, to become wrinkled, an appearance normally associated with higher animals such as cats and humans. Consequently, the bigenic mouse models of the invention can be used to study regulation of brain size and density as well as cellular composition and thereby provide a system for testing therapeutic agents that can reverse the neuronal deficit seen in patients with neurodegenerative diseases. The expanded zone of neural growth have cell precursors may also be a useful tissue for obtaining neural stem cells for therapeutic purposes.

The bigenic animal system of the present invention is further suited for analysis of medulloblastoma. For example, an inducible *RU486-GAL4* transcriptional activator that relies on *Pax-2* enhancer to target hedgehog protein expression to the cerebellum ensures that over-expression occurs after early brain development. This system avoids the abnormal development of the early brain that is observed in response to over-expression of hedgehog, and consequently enables formation of a cerebellum prior to activation of the regulatory protein.

The bigenic mouse model of the invention provides a means for modulating hedgehog gene expression for analyzing the effect of varied amounts of hedgehog, and an assay system for testing chemicals and cells as agents for therapeutic benefit for patients having abnormal hedgehog protein or suffering from the effect of abnormal levels of hedgehog.

Methods and Uses

The methods that are embodiments of the invention have utility in analyzing any of the components of hedgehog signaling pathways (Figure 1), both in terms of the biological activity of the individual components, and methods of modulating the activity of these compounds.

Screens for agents that reverse an ectopic hedgehog expression phenotype

An object of an embodiment of the present invention is the use of methods of assay with the bigenic animal and cell lines, mutants, vector constructs, and methods of the present invention, to identify, for example, the effect of misexpression of a target transgene. The misexpression of the transgene can be that of a reporter transgene, for example, a reporter gene on a vector that causes a color development such as *lacZ* as described in the examples herein. Methods of screening, including synthesis of chemical libraries and culture and assay of screen organisms in sterile multi-well plastic dishes containing for example 96 or 354 wells per dish, robots for delivery of samples to each well using devices such as automated multi-pipeters, and for processive manipulation of each dish, and for computerized reading of growth as optical density or production of light absorbant material at a given wavelength in each well, are well known to those of ordinary skill in the art of design of screens of chemical agents. The methods that are embodiments of the invention herein are suitable for automated and robotic applications, for example, by use of solvent systems to soluble a colored reaction product or product

capable of absorption of light of a particular wavelength. Programs for collection, analysis, storage and retrieval of assay data for each animal or cell line and vector at each condition of development and post-harvest incubation are available.

Such methods can be used to monitor potential inhibition of proliferation of tissues or cells of a bigenic animal in the presence and absence of a variety of chemical entity agents, and to record the extent of proliferation of cells and/or tissue differentiation in the presence and absence of the candidate chemical, and of control animal or cells under these conditions.

Pharmacological Methods

An agent, for example a hedgehog composition or analog or peptidomimetic, identified by an embodiment of the invention that is a method of assay can be subjected to a pre-clinical trial in an animal or a subject. For such a method, a "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, e.g., human albumin or cross-linked gelatin polypeptides, coatings, antibacterial and antifungal agents, isotonic, e.g., sodium chloride or sodium glutamate, and absorption delaying agents, and the like that are physiologically compatible. The use of such media and agents for pharmaceutically active substances is well known in the art. Preferably, the carrier is suitable for oral, topical, intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound can be coated in a material to protect the compound from the action of acids and other natural conditions that can inactivate the compound. Routes of administration also include, without limitation, intrauterine, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Administration of an agent to a maternal parent of an embryo progeny animal is within the scope of the invention.

Dosage regimens are adjusted to provide the optimum desired response, *e.g.*, a therapeutic response, such as restoration of a normal CNS proliferative response. For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced and administered over a time period by infusion, or increased, as indicated by the exigencies of the therapeutic situation.

One of ordinary skill in the art can determine and prescribe the effective amount

of the pharmaceutical composition required. For example, one could start doses at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a composition of the invention will be that amount of the composition which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application, are hereby expressly incorporated by reference.

Examples 1-3 describe materials and methods used in preparation of a bigenic mouse system which is capable of expressing a lethal mutation in the hedgehog protein. Example 1 describes construction of the vectors used in producing the parental transgenic mice, the genotype characterizations of which are described in Example 2. Procedures for analysis of mouse tissues by in situ hybridization and immunostaining are given in Example 3. Methods of measuring cell proliferation and apoptosis are given in Example 4, and the results observed show that misexpression of sonic hedgehog from 9.5 to 18.5 dpc caused hyperplasia of the embryonic CNS when *Shh* was under *GAL4*/UAS control.

A lethal phenotype was observed in Example 5 to result from micro-injection of the *Wnt-1* enhancer-mouse *Shh* transgene into mouse embryos. In Example 6, the phenotypes of progeny from a cross of parental lines was examined, and 25% (the bigenic progeny) were found to have a lethal phenotype characterized by the presence of an open neural tube in the midbrain region. Example 7 describes construction of a knock-out mutation of Indian hedgehog is constructed. In Example 8, the lethal phenotype of mouse embryos carrying an Indian hegehog null mutation was found to be due to a defect in formation of blood or blood supply.

Examples

Example 1. Construction of vectors for forming transgenic parents of bigenic animals

An overall strategy of the embodiment of the invention comprising bigenic animals is first constructing each of the two transgenic parents, then crossing them as shown in Figures 2-3 to obtain bigenic animals in the Mendelian proportion of one-quarter of the progeny. Vectors were constructed so that one transgenic parent contains a

driver vector which expresses an extra-mammalian transcriptional activator, and the other parent contains a vehicle vector capable of expressing a target gene in the presence of the transcriptional activator.

To construct these vectors, strategy of adding or removing gene sequences was utilized in forming the *wnt-1-GAL4* vector and the pUAS-*Shh* transgene vectors. As described below, pWEXP-2 (containing *wnt-1*) and pGaTB (containing *GAL4*) were combined to produce the tissue specific transcriptional activator vector. A second transgene vector carrying UAS-*Shh* was formed by inserting pUAS-shuttle into pWEXP3C-*Shh* and removing the *wnt-1* enhancer, resulting in a pUAS-*Shh* product.

(a) Constructing a vector containing a transcriptional activation sequence.

The plasmids pGaTB and pUAST encoding, respectively, full length *GAL4* and a pentamer array of its cognate DNA binding sequence, and the "upstream activating sequence" (UAS, 5'-CGG AGT ACT GTC CTC CG-3'; SEQ ID NO:1), were combined (Brand et al, 1993). To generate the transgene pWEXP*GAL4*, the *Wnt-1* expression vector pWEXP-2 (Echelard et al., 1993) was digested with *NruI* and treated with calf intestine alkaline phosphatase (CIAP). The plasmid pGaTB was digested with *Hind*III and *Fsp*I to release a DNA fragment encoding *GAL4*; this was end-filled with the Klenow fragment of DNA polymerase I and cloned into the pWEXP-2 vector. The transgene was purified from vector sequences by digestion with *Aat*II.

(b) Constructing a vector carrying a hedgehog gene regulated by an extra-mammalian promoter.

To generate the transgene pUAS-*Shh*, a shuttle vector, pUAS Shuttle, was constructed as follows. A *Kpn*I and *Bgl*II fragment of plasmid XB3 was replaced with an oligonucleotide polylinker encoding an *Xho*I site. This construct was digested with *Not*I and *Kpn*I, and was treated with CIAP. To this vector a *Not*I-*Kpn*I fragment of pUAS-*lacZ*, comprising five copies of UAS, was added, generating plasmid pUAS-Shuttle. Finally, pUAS-Shuttle was digested with *Xho*I and *Bgl*II and was treated with CIAP. A *Sal*I-*Bgl*II fragment of pWEXP-3C was isolated and cloned into the vector, creating plasmid pUAS-*Shh*. The transgene was purified from vector DNA by digestion with *Sal*I and *Bgl*II prior to micro-injection.

DNA sequencing of these constructs was carried out using both the ABI dye terminator and the di-deoxy chain termination methodologies. The genotyping

determinations for pWEXP2-*GAL4*, pWEXP3C-*Shh*, and UAS-*Shh* mice and embryos employed an upstream oligonucleotide primer from exon 1 of untranslated sequence of *Wnt-1* prier (5'-TAA GAG GCC TAT AAG AGG CGG-3'; SEQ ID NO:2), which primes approx. 60 bp upstream of the *Wnt-1* translational initiation site; a downstream primer from within *GAL4* (5'-ATC AGT CTC CAC TGA AGC-3', product size ca. 600bp; SEQ ID NO: 3); or mouse *Shh* (5'-CTC ATA GTG TAG AGA CTC CTC-3', product size ca. 600bp; SEQ ID NO:4). The following PCR conditions were used: 30sec, 93°C, 30sec, 55°C, and 1min, 72°C for 40 cycles; then 5min, 72°C.

(c) Constructing a vector carrying a reporter gene regulated by an extra-mammalian promotor.

To generate the reporter transgene pUAS-*lacZ*, the plasmid XB3 (Echelard et al., 1994) was digested with *Not*I and treated with CIAP. The pentamer array of UAS sequences from plasmid pUAST was amplified by PCR using primers that incorporated *Not*I and *Eag*I recognition sequences. The PCR products were digested and cloned into the XB3 vector to produce pUAS-*lacZ*. The transgene was purified from vector DNA by digestion with *Sal*I, and was micro-injected into control mouse embryos to confirm the specificity of gene expression under the tissue specific enhancer.

(d) Constructing a vector carrying a hedgehog gene regulated by an extra-mammalian promoter.

To generate the mouse Sonic hedgehog misexpression transgene pWEXP3C-*Shh*, the full length cDNA of *Shh* was digested from plasmid 6.1 (Echelard, et al., 1993) with the enzymes *EcoR*1 and *Spe*1, end-filled and cloned into vector pWEXP-3C (Danielian P. et al. (1996) Cell 75:1417-1430) using standard splicing and ligation reactions. The transgene pWEXP3C-*Shh* was purified from vector DNA by digestion with *Sal*I, and was micro-injected into control mouse embryos to determine that the *Shh* gene encoded the correct functional protein and caused the lethal phenotype.

Example 2. Production and genotyping of transgenic mice

Transgenic mice were generated by micro-injection of linear DNA fragments obtained from vector DNA into pronuclei of B6CBAF1/J (C57BL/6J x CBA/J) zygotes as described (Echelard et al., 1994). The transgenic mouse line *Wnt-1/GAL4* was produced by injection of transgene pWEXP2-*GAL4*. All transgenic mice were made following standard protocols as described in "Manipulating the Mouse Embryo" B. Hogan, 2nd Ed.

Cold Spring Harbor Press, Cold Spring Harbor, NY (1994).

Founder (G_o) transgenic mice were identified by Southern blot of *EcoR*I-digested genomic DNA using probes for *GAL4* (line WEXP2-*GAL4*) or *lacZ* (lines UAS-*lacZ* and UAS-*Shh*; probes described in Rowitch et al., 1999, *Devel. Neurosci.*, in press, incorporated herein by reference). Subsequent genotyping of UAS-*lacZ* transgenic embryos or mice by PCR was carried out as described in Echelard, et al. (1994). Genotyping of WEXP2-*GAL4* and UAS-*Shh* transgenic embryos or mice was performed using the upstream primer described supra (SEQ ID NO:2) from exon 1 of the untranslated sequence of *Wnt-1* and the downstream primer described supra (SEQ ID NO:3) from within *GAL4* or mouse *Shh* (SEQ ID NO:4) , respectively. PCR conditions were as described in Echelard et al. (1994).

The transgenic mice carrying any one of the vectors that are embodiments of the invention exhibited normal wild-type phenotypes in comparison to parental mice which had not received a transgene.

Example 3. Whole mount and section histology, in situ hybridization and immunohistochemistry for assay of misexpression

Analysis of embryos for β-galactosidase activity was carried out as described by Whiting, J. et al. (1992) Genes Devel.5:2048-2059). For analysis of skeletal elements, 18.5 dpc bigenic embryos were processed as described by McLeod, J. et al. (1990) Teratol. 22:229-230.

For analysis of tissues by histological analysis or in situ hybridization, embryos were harvested between 9.5-18.5 dpc, dissected in phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde. Whole mount in situ hybridization was carried out per standard lab protocols. Embryos for histologic analysis, BrDU incorporation, and in situ hybridization, were fixed in either Bouin's or 4% paraformaldehyde overnight or up to 24 hrs, embedded in paraffin wax and sectioned at 6-7 μm. Sections were stained with hematoxylin-eosin or toluidine blue.

In situ hybridization on paraffin sections with radiolabeled anti-sense RNA probes was carried out on either paraformaldehyde or Bouin's fixed tissues according to Wilkinson, D. (1992) In situ hybridization: a practical approach. BRL Press. Darkfield photomicrographs were collected on a Leitz Orthoplan or Nikon E600 compound microscope using a 35 mm camera and Fuji Velva film or a SPOT I digital camera

(Diagnostic Imaging). In situ hybridization on frozen sections of paraformaldehyde-fixed tissues with digoxigenin labeled anti-sense probes was carried out as described in Ma, Q. et al. (1997) J. Neurosci.17:3644-3652, and photomicrographs were collected as above.

For immunohistochemistry, embryos were either fresh frozen or fixed between 6-24 hrs prior to freezing and cryostat sectioning. Photography of live embryos was carried out in PBS on an Olympus SZH10 microscope using Kodak 64T film, or a Nikon camera and daylight film, respectively.

Example 4. Misexpression of hedgehog causes excessive cellular proliferation

For analysis of proliferation, BrDU (Sigma, St. Louis, MO) incorporation was measured using a dose of 50 μg/kg injected intraperitoneally into pregnant mice exactly 3 hrs before sacrifice at 12.5 and 18.5 dpc. Embryos were fixed either in Bouin's or 4% paraformaldehyde and sectioned as described above. Dividing cells that had incorporated BrDU were identified using monoclonal IgG (Becton-Dickenson) and immunoperoxidase staining (Vector Labs; Burlingame, CA) employing FITC-tyramide (NEN; Boston, MA). Apoptotic death was measured by techniques established in the art, for example, the TUNEL procedure (Gavrieli, Y. et al.(1992) J.Cell Biol. 119:493-501) on adjacent sections, and electron microscopy. Reagents TdT and biotinylated-16dUTP were obtained from (Boehringer-Mannheim).

To investigate the embodiment of the invention comprising proliferative effects of *Shh* in the spinal cord, *Shh* was placed under *GAL4*/UAS regulation which gives consistent expression of *Shh* at ectopic locations such as the roofplate of transgenic mouse embryos. The phenotype observed to result from *Shh* misexpression included hyperplasia of the dorsal CNS, and activation of Hedgehog transcriptional targets, e.g., *Patched* and *Gli*, and was observed in embryos from 9.5-18.5 dpc (Figures 4-6).

Surprisingly, increased cellular proliferation as measured by BrDU incorporation was observed at 12.5 dpc but not at 18.5 dpc, despite continuous exposure to *Shh*. Hyperplastic tissues were predominantly nestin-positive, however, dispersion of tissue samples into cell culture medium yielded differentiation of cells into neurons, astrocytes and oligodendrocytes. Markers of ventral progenitor populations in the spinal cord were expressed with an altered pattern as a consequence of ectopic *Shh* expression.

Example 5. The effect of a lethal mutation in hedgehog protein

To determine whether the *Wnt-1* enhancer-mouse *Shh* transgene (WEXP3c-*Shh*,

which is not under *GAL4* regulation) was lethal, approximately 300 mouse embryos were injected and harvested at 10.5 dpc. The phenotype of all embryos that expressed this transgene was the presence of an open neural tube, indicating that the transgene was lethal, unlike the bigenic UAS-*Shh* mouse line animals having a UAS-*GAL4* dependent regulation above.

In one embodiment of a transgenic line of the invention, the yeast transcription factor *GAL4* was expressed in the dorsal CNS under the control of the Wnt-1 enhancer (WEXP-*GAL4*; Figure 3). A second transgenic line (UAS-*lacZ*) directed expression of reporter gene β-galactosidase (*lacZ*), under control of UAS (Figure 3). When the UAS-*lacZ* heterozygous animals were mated with WEXP-*GAL4* heterozygotes, 25% of progeny embryos showed β-galactosidase expression in the *Wnt-1* pattern of tissues and cells. The progeny capable of expressing β-galactosidase in the *Wnt-1* pattern were otherwise normal in phenotype.

This experiment demonstrates that *GAL4* was capable of functioning at its cognate DNA-binding sequence in cells of the developing CNS, that the pattern of gene expression with respect to time and cells of a tissue was programmed by *Wnt-1*, and that the vector system was capable of activating gene expression of a bacterial reporter transgene under the control of the UAS.

Example 5. The effects of sonic hedgehog misexpression in the embryonic CNS in bigenic mouse progeny

In order to study the effects of *Shh* misexpression in the embryonic CNS, a transgenic mouse line containing *Shh* under control of the UAS enhancer (UAS-*Shh*) was generated in Example 2. The UAS-*Shh* mouse line was observed to be stable, and animals showed no detrimental effects of *Shh*. However, when animals were crossed to mice from the WEXP-*GAL4* line, 25% of embryos were observed at 10.5 dpc to have each developed an open neural tube in the region of the midbrain (Figure 5, Panel E), as a result of ectopic expression of *Shh*. At 18.5 dpc, the gross phenotype of progeny of the UAS-*Shh* x WEXP-*GAL4* cross (referred to as *Shh*-Tg) was substantial hyperplasia of the dorsal brain and spinal cord, as well as hydromyelia (Fig.5). The phenotype was consistent among the progeny observed, indicating 100% penetrance of the *Shh* gene, i.e., that the phenotype and the genotype of animals coincided in every case, and was observed in at least 50 *Shh*-Tg progeny embryos.

Example 6. Construction of an Indian hedgehog loss of function mutation

The Indian hedgehog gene *Ihh* has been thought to be expressed in the yolk sac of visceral endoderm from 8 dpc (Farrington, S., et al. (1997) Mech.Dev. 62:197-211), in the gut epithelium lining mid-and hindgut from 10.5 dpc and in the hindstomach and columnar epithelium of inestine and rectum (Bitgood, M. et al. (1995) Dev. Biol. 172:126-138), in tooth dental lamina from 9.5 dpc (Kronmiller, J. et al. (1996) Arch.Oral.Biol.41:577-583), in the metanephros of the kidney from 14.5 dpc and in adult kidney and proximal tubules, in the retina of the eye (Jensen, A. et al. (1997) Devel.124: 363-371), in chondrocyte cartilage nodules from 11.5 dpc and in maturing chondrocytes overlalpping with proliferative and hypertrophic zones (Bitgood et al., 1995), and in osteoblast cell lines and osteoblasts enriched cultures from neonatal rat calvariae (Murakami, S. et al. (1997) Endocrinol. 138:1972-1978).

No homozygous mutants have been isolated or constructed, however, to permit further analysis and identification these presumed *Ihh* tissue targets. To determine whether *Ihh* is an essential gene, i.e., whether a potential lethal phenotype is demonstrated by an *Ihh/Ihh* homozygotes, a targeting vector (Figure 8) based on the structure and restriction sites of the *Ihh* gene was constructed. The targeting vector carries a DNA with a deletion of the E1 of *Ihh* replaced by *neo*, and having DNA encoding TK following E2 and E3 of the *Ihh* gene.

Following successful isolation of mice carrying the markers of the targeting vector, probes for distinguishing the wild type and insertion knock-out mutant *Ihh* genes by digestion and analysis of *Xho*I and *Nco*I fragments were used, and the predicted sizes of *Xho*I and *Nco*I fragments that hybridized with the probes and that were used to identify each genotype, are also shown in Figure 8. Mice carrying the *Ihh* knockout gene, and heterozygous for the normal *Ihh* gene were thus constructed.

Example 7. Crossing knock-out heterozygotes to determine the effects of an Indian hedgehog loss of function mutation among homozygous progeny

Mice carrying the knockout mutation constructed in the previous example were bred, and embryos having each of a homozygous wild type genotype, a heterozygous genotype, and a homozygous mutant *Ihh* genotype were harvested from among all embryos as a function of time after the matings. It was here observed that the significant loss of the embryos having the homozygous mutant *Ihh* genotype, observed as an overall

deviation from the Mendelian ratio of 1:2:1, indicated that embryos of this class have substantially less viability from between 10.5 to 12.5 dpc (Table 1). The data in Table 1 indicate that loss had occurred of a significant fraction of the embryos of the homozygous mutant class of progeny, compared to the stable numbers of embryos observed of wild-type and heterozygote classes over this time period (maintained as a stable ratio of homozygous wild type to *Ihh* heterozygotes of 1:2). A dramatic loss in viability of *Ihh* mutant homozygotes occured after 10.5 dpc with further decline occurring also after this time point.

Embryonic death of *Ihh* homozygotes was determined to be due to a defect in formation of the blood or the blood supply. A further aspect of the phenotype was a reduction in the size of the main blood vessels observed in the null double *Ihh* mutant embryos compared with normal embryos (homozygotes and heterozygotes) at 11.5 dpc. These results show that a knockout system utilizing the *Ihh* gene provides a useful model for the study of vasculogenesis in a mammalian animal.

Example 8. Collagen II enhancer from early chondrocytes

Bigenic mice were formed by crossing a first parental mouse line having a driver vector with the collagen II enhancer (*col II*; Metsarante, M. et al. (1995) Dev. Dyn. 204:202-210) used to express *GAL4*, with a second mouse line having a carrier vector with UAS upstream of *Ihh*. Expression in the embryonic progeny of this cross was compared to that of progeny from crossing the first parent with each of UAS-*lacZ* and UAS-*Shh*. The data show that expression of *GAL4* under control of the collagen II enhancer restricts expression to chondrocytes, and further that *Ihh* expression can be specifically activated in chondrocytes.

These data, in combination with data obtained from using *Wnt-1-GAL4* to cause expression specifically in the CNS show that it is possible to combine different transgenic animal lines to achieve new combinations of gene expression in bigenic progeny animals.

Genotypes of embryos from *lhh* +/- intercrosses

genotypes	10.5 dpc	12.5 dpc	14.5 dpc	16.5 dpc	18.5 dpc	total
+/+	11	14	21	21	21	88 (29.5%)
+/-	25	33	42	49	27	176 (59.1%)
-/-	12 (25%)	7 (13%)	6 (8.7%)	4 (5.4%)	5 (9.4%)	34 (11.4%)
total	48	54	69	74	53	298

Table 1

What is claimed is:

1. A transgenic non-human mammal, substantially all of whose cells contain a non-viral regulatory DNA sequence linked to a recombinant hedgehog gene introduced into the mammal or an ancestor of the mammal at an embryonic stage.

2. A transgenic non-human mammal according to claim 1, the mammal having an endogenous coding sequence substantially the same as a coding sequence of the recombinant hedgehog gene.

3. A transgenic non-human mammal according to claim 2, wherein the mammal is a rodent.

4. A transgenic non-human mammal according to claim 3, wherein the rodent is a mouse.

5. A transgenic non-human mammal according to claim 1, wherein the regulatory sequence comprises a UAS sequence (SEQ ID NO:1).

6. A bigenic non-human mammal, substantially all of whose cells contain a non-viral regulatory DNA sequence linked to a recombinant hedgehog gene sequence; and a transcriptional activator sequence, introduced into the mammal or an ancestor of the mammal at an embryonic stage.

7. A bigenic non-human mammal according to claim 6, having an overexpressed vascular system in the central nervous system.

8. A bigenic non-human mammal according to claim 7, wherein the mammal is an embryo.

9. A bigenic non-human mammal according to claim 8, wherein the mammal is a mouse and is capable of a lifespan of at least 9 dpc.

10. A bigenic non-human mammal according to claim 6, wherein the transcriptional activator gene is *GAL4*.

11. A bigenic non-human mammal according to claim 6, wherein the transcriptional activator gene is regulated by a tissue specific promoter.

12. A bigenic non-human mammal according to claim 6, wherein the tissue specific promoter is a *wnt* promoter.

13. A bigenic non-human mammal according to claim 6, wherein the tissue specific promoter is a *col II* promoter.

14. A bigenic non-human mammal according to claim 6, wherein the transcriptional activator gene is regulated by an inducible promoter.

15. A bigenic non-human mammal according to claim 14, wherein the inducible promoter is regulated by a fusion of *GAL4* protein and a second protein.

16. A bigenic non-human mammal according to claim 15, wherein the second protein is activated by binding an RU486 mifepristone molecule.

17. A bigenic non-human mammal according to claim 6, for use as a model for disease.

18. A bigenic non-human mammal according to claim 17, wherein the disease is cancer.

19. A bigenic non-human mammal according to claim 18, wherein the cancer is selected from the group consisting of a cancer of the breast, skin, prostate, kidney, lung, and central nervous system.

20. A bigenic non-human mammal according to claim 18, wherein the cancer

is a primitive neuroectodermal tumor.

21. A bigenic non-human mammal according to claim 19, wherein the cancer is a medulloblastoma.

22. An isolated cell of a bigenic non-human mammal obtained from the mammal of claim 6.

23. An isolated cell of the bigenic non-human mammal according to claim 22, selected from the group consisting of an embryonic-stem cell, a tumor cell, a nerve cell, and a vascular cell.

24. A transgenic non-human mammal having an insertion mutation of an *Ihh* gene.

25. A transgenic mammal non-human according to claim 24, wherein the insertion comprises a selectible marker.

26. A transgenic mammal non-human according to claim 25, wherein the insertion comprises a deletion of the *Ihh* gene.

27. An isolated population of cells selected from the group consisting of a transgenic non-human mammal according to claim 1, and its bigenic progeny.

28. A method of identifying the effect of misexpression of a target transgene in a signal transduction pathway that includes a hedgehog protein, in a progeny animal, comprising:

(a) forming a first transgenic animal having a first transgene encoding a transcriptional activator of a eukaryotic species different from the animal;

(b) forming a second transgenic animal having a second transgene comprising the target gene and having a recognition sequence for the transcriptional activator that is located upstream of the target gene;

(c) mating the first and the second transgenic animals to form a bigenic animal; and

(d) causing the target gene to be misexpressed in the animal.

29. A method of identifying the effect of misexpression of a target transgene according to claim 28, wherein the transgenic animals are formed from an animal which is an animal model disease line.

30. A method of identifying the effect of misexpression of a target transgene according to claim 28, wherein the animal model is selected from the group consisting of a cancer and an autoimmune disease.

31. A method of assaying for a temporal requirement for the presence of a hedgehog protein on progression of a disease, comprising:

(a) forming a bigenic animal according to the method of claim 28;

(b) treating the bigenic animal for an effective time interval with an agent that interrupts the hedgehog pathway; and

(c) assaying the progression of the disease in the animal in (b) compared to the progression of the disease in the animal in (a).

32. A method of assaying for a temporal requirement for the presence of a hedgehog protein during progression of the disease according to claim 31, wherein the treatment comprises administration of an agent selected from the group consisting of an inhibitor of cholesteroid biosynthesis, an anti-hedgehog antibody, and a sterol analog.

33. A method for determining therapeutic efficacy of an agent, comprising:

(a) forming a bigenic mouse according to claim 28, wherein the misexpressed target gene is a hedgehog gene;

(b) administering the agent to the mouse; and

(c) determining therapeutic efficacy of the agent.

34. A method according to claim 33, wherein in (b) further comprises

administering the agent to the mouse in a pharmaceutical carrier at an effective dose.

35. A method according to claim 34, wherein (c) further comprises comparing lifespans of the bigenic mouse of (b) with the bigenic mouse of (a).

36. A method according to claim 29, wherein the bigenic mouse in (a) is an embryo.

37. A method of obtaining an expanded population of neural stem cells from a subject, comprising:

treating a neural stem cell from the subject with a hedgehog protein, so that proliferation of the stem cell provides an expanded population of neural stem cells.

38. A method according to claim 37, wherein the hedgehog protein is sonic hedgehog protein.

39. A method according to claim 37, wherein the subject has a condition selected from the group consisting of Parkinson's disease, Alzheimer's disease, and spinal cord injury.

40. A method according to claim 39, wherein the condition is treated by administration to the subject of a sample of the expanded cell population.

41. A method for inactivating an *Ihh* gene in a non-human mammal, comprising:

(a) constructing a recombinant vector carrying an *Ihh* insertion mutation;

(b) injecting an embryonic stem cell with the vector; and

(c) implanting the stem cell into an adult mammal.

42. A method for inactivating an *Ihh* gene according to claim 41, wherein the vector in (a) carries a deletion of exon 1 of the *Ihh* gene.

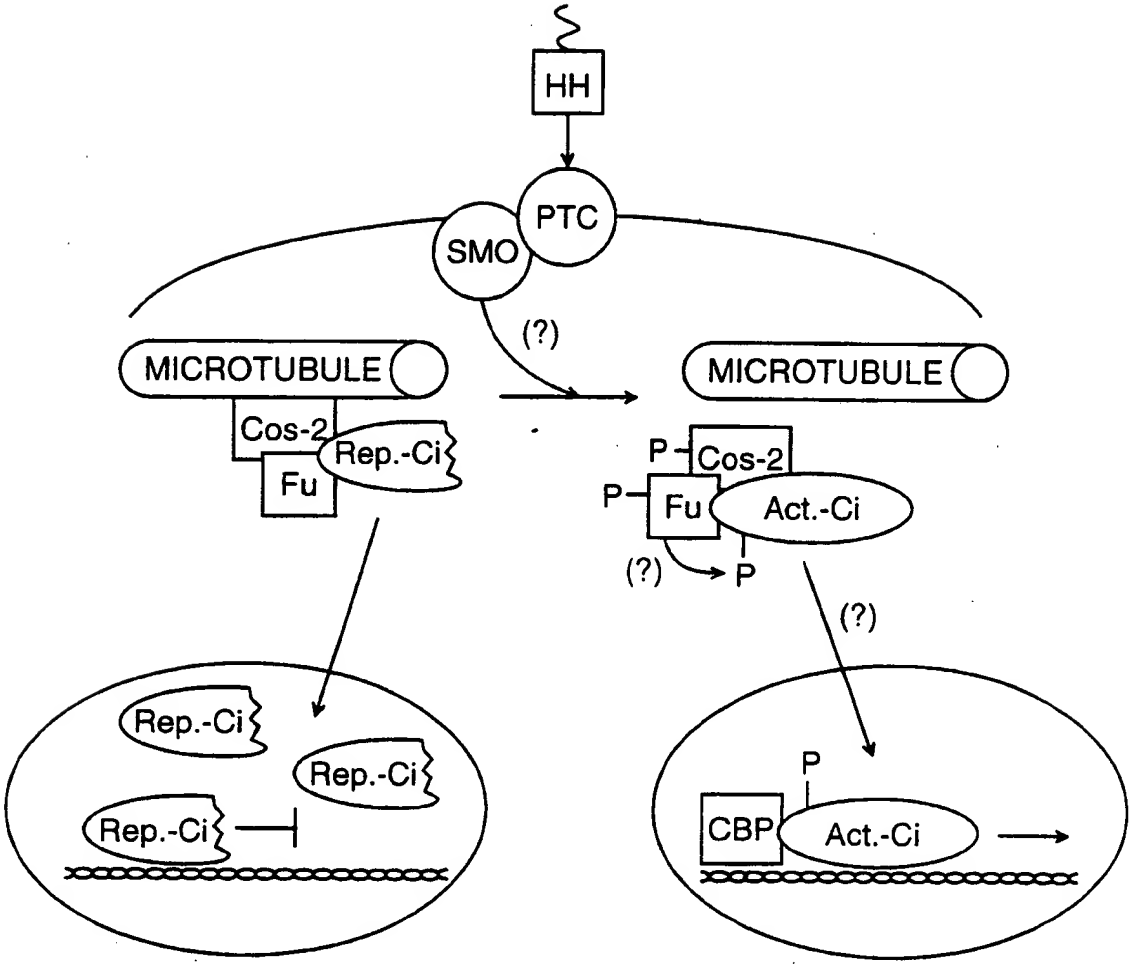

HH
PTC
SMO
(?)
MICROTUBULE
Cos-2
Fu
Rep.-Ci
MICROTUBULE
P
Cos-2
P
Fu
Act.-Ci
(?)
P
(?)
Rep.-Ci
Rep.-Ci
Rep.-Ci
P
CBP
Act.-Ci
Nucleus:
no HEDGEHOG
Nucleus:
after HEDGEHOG signaling

FIG. 1

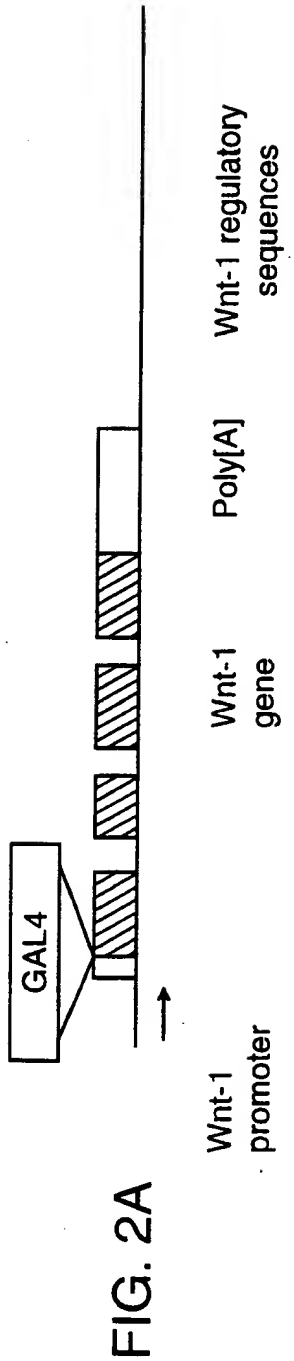


FIG. 2A

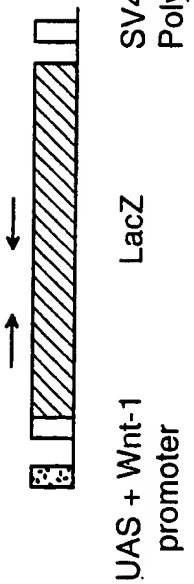


FIG. 2B

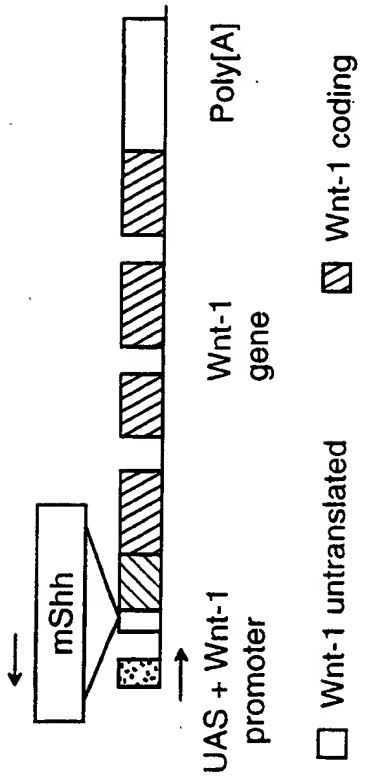


FIG. 2C

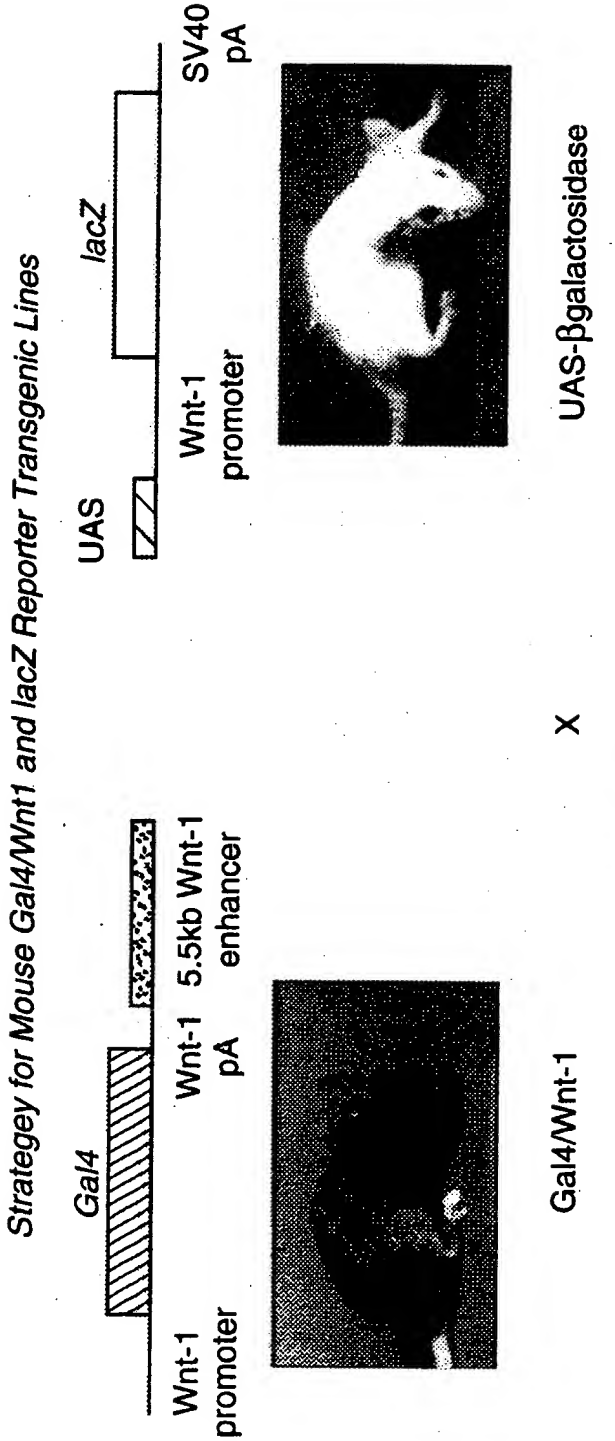
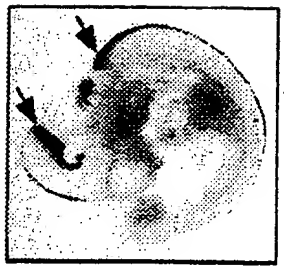

Strategey for Mouse Gal4/Wnt1 and lacZ Reporter Transgenic Lines
Gal4
Wnt-1 promoter
Wnt-1 pA
5.5kb Wnt-1 enhancer
Gal4/Wnt-1
X
UAS
lacZ
Wnt-1 promoter
SV40 pA
UAS-βgalactosidase

FIG. 3



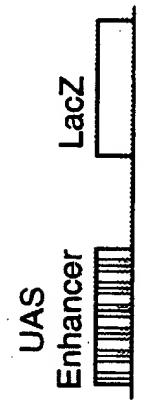
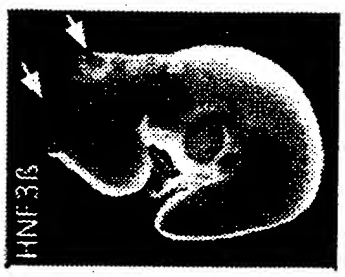


FIG. 4B



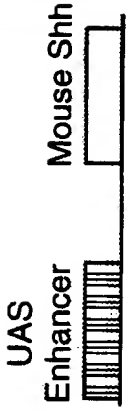


FIG. 4C

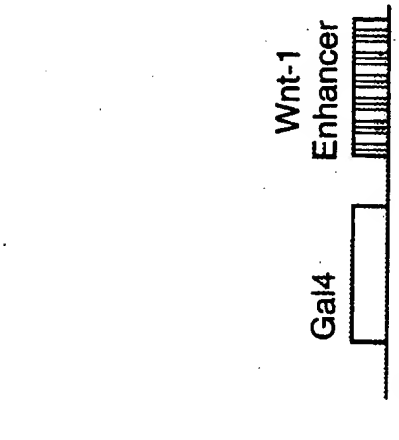


FIG. 4A

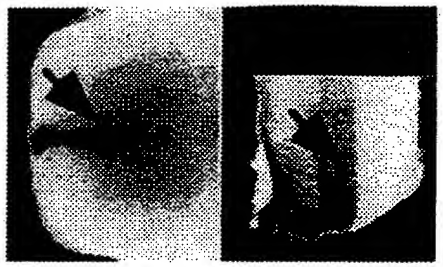


FIG. 5D

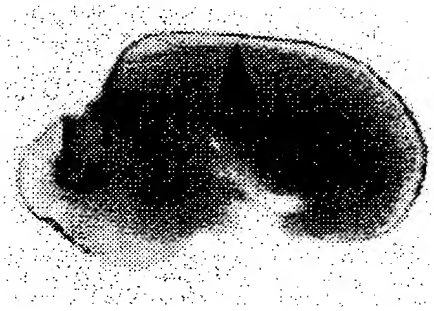


FIG. 5C

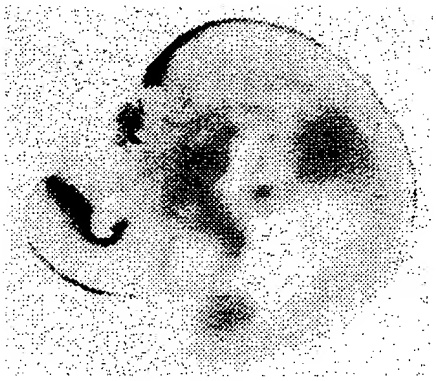


FIG. 5B

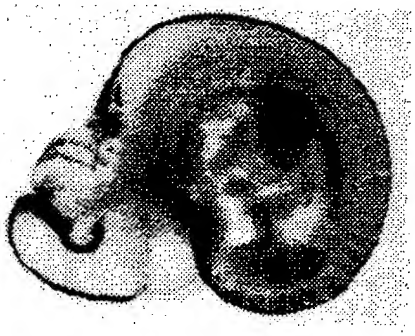


FIG. 5A

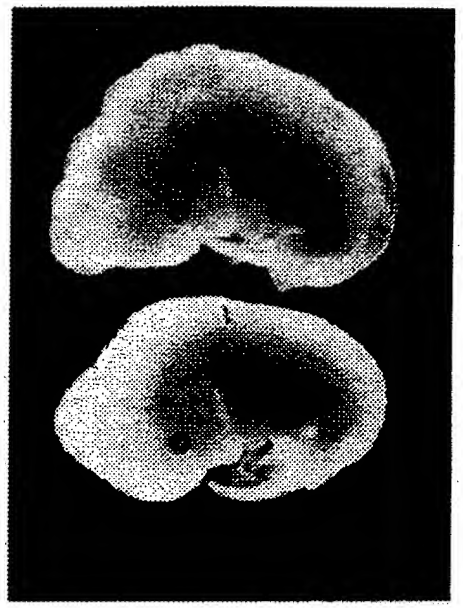


FIG. 5F

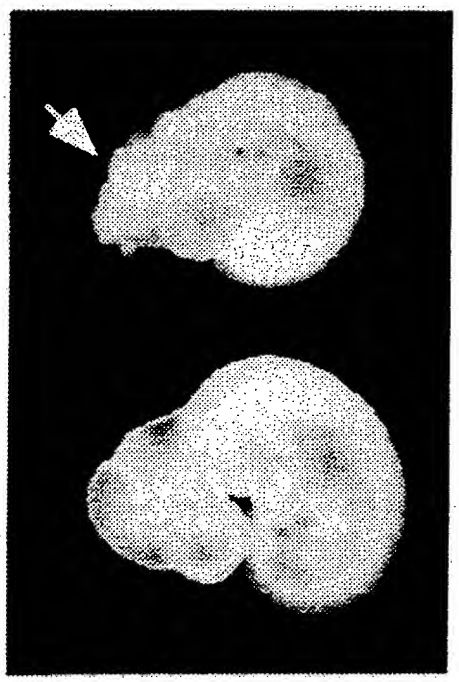


FIG. 5E

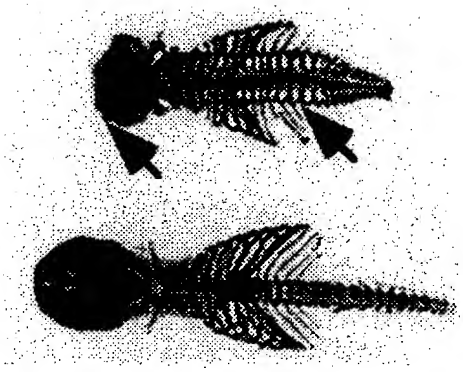


FIG. 5J

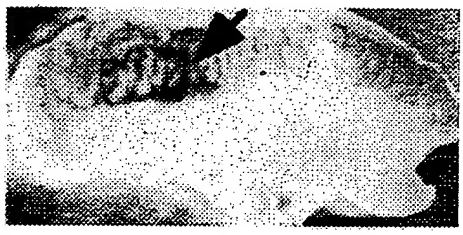


FIG. 5I

FIG. 5H

FIG. 5G

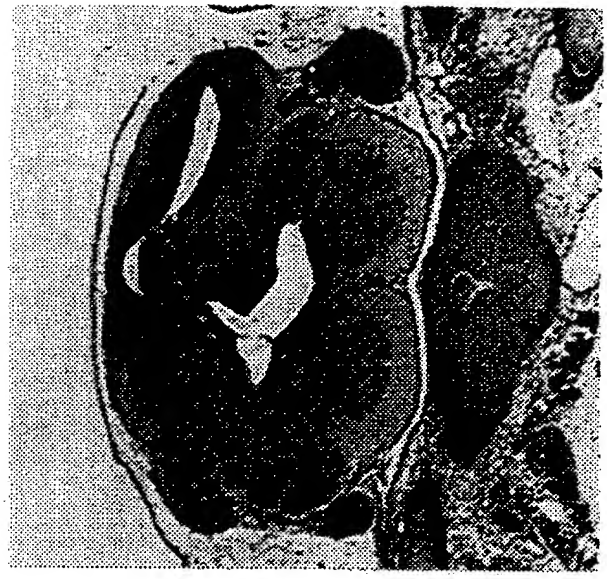


FIG. 6B

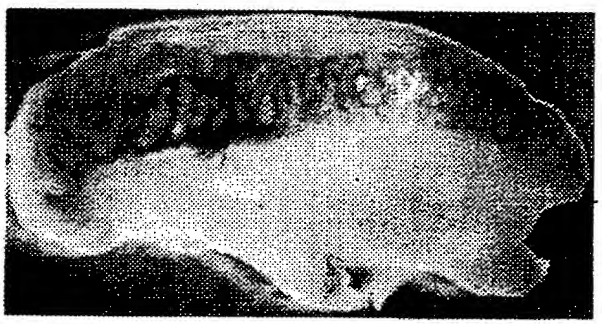
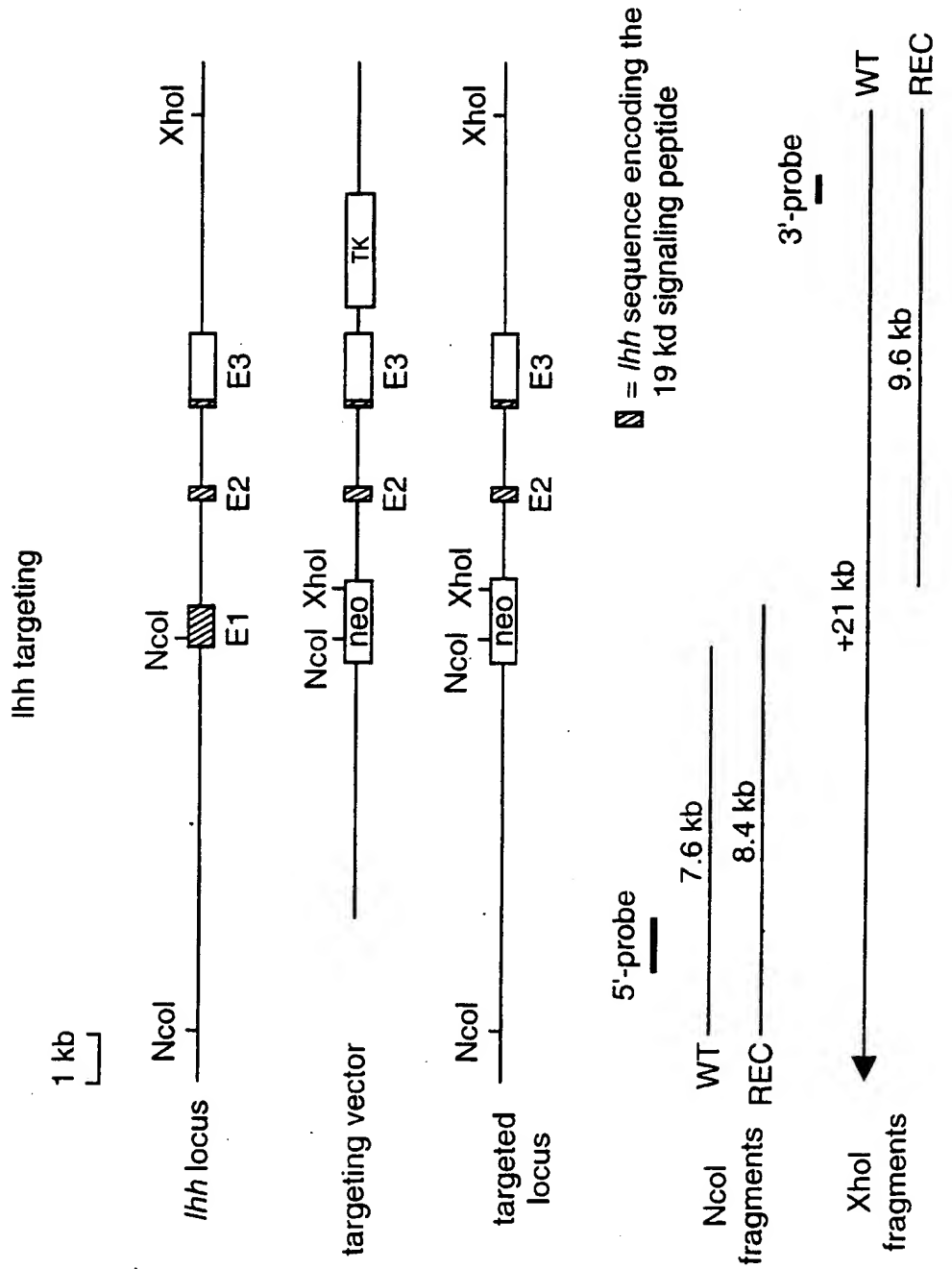


FIG. 6A

Phenotype of UAS-Shh Transgenic Mice at E10.5
Gal4
Wnt-1 promoter
Wnt-1 pA
5.5kb Wnt-1 enhancer
X
UAS
Wnt-1 promoter
Mouse Shh
SV40 pA
Whole mount HNF-3β immunostaining
mb
hb
ectopic dorsal HNF-3β
floor plate
dorsal view

FIG. 7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1 kb
NcoI
Ihh locus
NcoI
E1
E2
E3
XhoI
NcoI XhoI
neo
TK
targeting vector
NcoI
targeted locus
= Ihh sequence encoding the 19 kd signaling peptide
5'-probe
7.6 kb
WT
NcoI fragments
8.4 kb
REC
3'-probe
+21 kb
WT
9.6 kb
REC
XhoI fragments

FIG. 8

SEQUENCE LISTING

<110> Harvard University
David H. Rowitch
Andrew P. McMahon

<120> Use of Bigenic Mouse Models and Assay Systems to Identify Agents that Regulate Proliferation and Differentiation

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